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Partial sequence determination of a cDNA encoding microneme 5 protein of *Eimeria necatrix* isolated in Khuzestan province, Iran

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Abstract

Micronemes are secretory organelles of the invasive stages of apicomplexan parasites and contain proteins that are important for parasite motility and host cell invasion. Even though coccidiosis is a complex disease that can be caused by any combination of mainly seven species, most of the molecular researches concerning characterization of host and parasite genes and proteins have been limited to *Eimeria tenella*. The present study describes isolation and purification of *Eimeria necatrix* oocysts that can be used for an inexpensive and simple total RNA extraction method to partial sequence determination of a cDNA encoding microneme 5 protein. Using the extracted total RNA as template and oligo(dT) as primer, cDNA was synthesized. In order to amplify cDNA encoding the micronem 5 protein (EnMIC-5), RT-PCR was applied with the specific primers based on the known EST sequence. Following amplification, the unique and thick 758 bp fragment was seen. Domain analysis of EnMIC-5 revealed that the sequence contains the conserved domain of PAN/APPLE superfamily between amino acid resides 130 to 201. This domain has strong similarity to the adhesive plasma pre-kallikrein. Despite sequence similarity of EnMIC-5 with those sequences in database, differences may represent some allelic polymorphism.

Keywords: ***Eimeria necatrix*,** Microneme, RT-PCR

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Introduction

Eimeria species are the most important protozoan parasites of poultry and many other domestic livestock. An economic loss due to this parasite has estimated to be up to £1500 million worldwide per annum (Shirley *et al.*, 2005).

Eimeria are obligatory parasites (sporozoa) belonging to the phylum Apicomplexa characterized by the presence of an apical complex (micronemes, rhoptries and dense granules) in the free stages of the cycle (sporozoites and merozoites) which attribute to invasion to the epithelial cells (Badran and Lukeshova, 2006). The soft bodied parasites are safely encapsulated inside an extremely robust structure called oocyst that is resistant to mechanical and chemical damage (Ayub Ali *et al.*, 2009).

Eimeria tenella and *Eimeria necatrix* are the most pathogenic species among seven species of *Eimeria* causing coccidiosis in chickens (Shirley, 1995). *E. necatrix* causes spectacular lesions in the small intestine where the non sexual stages develop but the sexual stages leading to form oocyst occur in the ceca (McDougald, 2003).

As for other apicomplexan parasites, understanding of the interactions of *Eimeria* with their hosts and environments are far from complete, although during the recent years, a major goal of basic researches on *Eimeria* species has been identifying candidate proteins for developing a subunit recombinant vaccine to control avian coccidiosis. Sequencing of genes for organelle proteins has shown several domains and motifs conserved among genera, especially in microneme proteins, supporting the idea that apical proteins have similar functions across the phylum (Tomley, 1997). A microneme protein of *E. tenella* (EtMIC-5) which has eleven cysteine-rich receptor-like regions with striking similarity to the Pan/Apple domains of the binding regions of blood coagulation factor XI and plasma pre-kallikrein has been reported (Brown *et al.*, 2000). Putative Pan domains are also found in micronemal proteins of *Sarcocystis muris* (Klein *et al.*, 1996) and *Toxoplasma gondii* (Brecht *et al.*, 2001). The majority of micronemes comprise multiple copies of a limited number of adhesive domain types, which has allowed the identification of a large number of additional putative microneme proteins bearing these domains in the parasite databases. Based on this it is likely that many more proteins will be shown to occupy the micronemes in future studies (Carruthers and Tomley, 2008).

The discovery of these antigens for *Eimeria* species has been partially hampered due to the fact that the bulk of research concerning gene and antigen discovery using expressed sequence tag (EST) analysis, genomics, and proteomics has been limited to *Eimeria tenella* (Li et al., 2003 and Refeaga *et al.,* 2003 and Miska *et al.,* 2004 and De Venevelles *et al.,* 2006). *E. necatrix* has low reproductive capability and infects different portions of the intestine than *E. tenella*. Therefore, recovery of the oocysts and intracellular stages of *E. necatrix* is more difficult. This has led to a far larger body of molecular data being generated for *E. tenella*. For example, 34,000 ESTs from *E. tenella* have been deposited in the GenBank database, compared with 53 ESTs generated from *E. necatrix*. Analyzing ESTs from multiple species of *Eimeria* infecting chickens is important because it will help identify genes and proteins which sequences and expression are conserved. Taking advantage of an EST sequence analysis (EU335049), the present study was performed to determine the partial sequence of the microneme 5 protein of *E. necatrix* from an Iranian isolate.

Materials and methods

Isolation, purification and propagation of Eimeria necatrix: Eimeria oocysts were collected from cecal contents of chickens, slaughtered in Ahvaz abattoir. Primary identification of *E. necatrix* carried out by morphologic and morphometeric criteria and then by polymerase chain reaction (PCR), based on primers specific for ITS-1 (Internal Transcribed Spacer 1) region of 18 s rRNA

gene (Su *et al.*, 2003). For this purpose, DNA extraction was carried out in 300 µl of lysis buffer [0.1 M TRIS–HCl, 0.1 M NaCl, 50 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 5 mg/ml of proteinase K, pH 8], after an overnight incubation at 40°C (Zhao *et al.*, 2001). Thereafter, single sporulated oocysts were obtained according to the procedures described by Lee (1979) and inoculated orally to ten 3-week-old coccidia-free chickens .7 days later, pure oocysts were recovered from the ceca using either enzymatic or chemical treatments (Tomley ., 1997) and allowed to sporulate according to the procedures described by Shirley (1995) (Fig.2). Sporulated oocysts were purified from bacterial and fungal contamination by treatment with 5.75% sodium hypochlorite and incubation on ice for 30 min (Zhao *et al.*, 2001) followed by extensive washing with sterile cold water. The resulting pellet was mixed with saturated sodium chloride solution and the tube content was overlaid with distilled water. After centrifugation, the purified oocysts were collected from the interface of the salt and water and washed several times with cold water, rinsed with phosphate buffered saline (PBS) and used immediately.



Figure 1: Sporulated oocysts of *Eimeria necatrix* before breaking (4X)

Total RNA extraction: Total RNA was extracted from the sporulated oocysts using acid-phenol extraction procedure based on the method of Chomczynski and Sacchi (1987) with minor modifications. The isolation procedure exploits the ability of phenol and sodium dodecyl sulfate (SDS) to inhibit RNase activity (Farrell 1993) and that of lower pH to protect RNA from base-catalyzed hydrolysis. Consolidation of an extraction buffer prepared by mixing of water-saturated phenol with an acidic solution of sodium acetate causes DNA to be extracted out of the aqueous phase and into the interphase and organic phase. RNA may therefore be recovered from the aqueous phase. Briefly, 5×10^5 of oocysts were added to 500 mg of glass beads in a 50ml disposable polypropylene tube. The reagents were then added in the following order: 500 µl of TRIS-ethylene-diamine tetraacetic acid (TRIS-EDTA) pH 8.0, 500 µl of water-equilibrated phenol, 100 µl of 3 M sodium acetate (pH 5.2), and 50 µl of 20% SDS. The equilibration of phenol with water rather than buffer is necessary to maintain an acidic pH. The mixture was vortexed for 4 × 1 min in alternation with 1-min incubations on ice. Breakage of the oocysts was monitored microscopically until no intact oocysts, sporocysts and also sporozoites were seen (Fig.2). The resulting suspension was pipetted off the glass beads and transferred to microcentrifuge tubes. An equal volume of water-equilibrated phenol was added to each tube, mixed, and centrifuged in an Eppendorf microcentrifuge at 14,000 rpm for 30 sec at 4 °C. The aqueous layer was removed to a new microcentrifuge tube and extracted twice more with water-equilibrated phenol and once with chloroform as outlined above. The final aqueous layer was removed and the RNA was precipitated with 0.1 vol. of 3 M potassium acetate (pH 5.2) and 2.5 vol. of 95% ethanol. The RNA was allowed to precipitate for 2 h at -20°C. The RNA was pelleted by centrifugation at 14,000 rpm for 10 min at 4 °C, washed in 70% ethanol, air-dried, dissolved in sterile double distilled

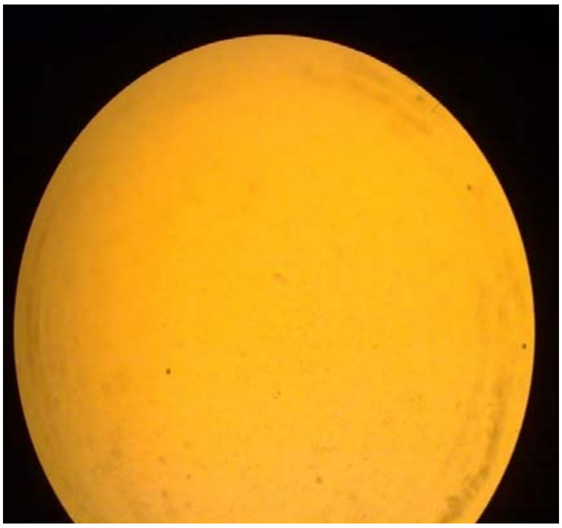


Figure 2: Sporulated oocysts of *Eimeria necatrix* after breaking (4X)

diethylpyrocarbonate (DEPC)-treated water and was measured spectrophotometerically at λ_{260} and used for cDNA synthesis immediately.

cDNA synthesis: The complementary DNA (cDNA) was synthesized with the extracted total RNA as template and oligo(dT) as primer. To synthesis the cDNA, 0.5μg of Mod-T primer was added to the extracted total RNA (3 µg) and incubated at 70°C for 10 minutes followed by a brief centrifugation. The reaction was chilled on ice for a few minutes and then added 1 µl Protector RNase Inhibitor (Roche, Switzerland), 1 µl dNTP mixture (Cinnagen, Tehran, Iran) (120 mM of each nucleotide), 2.5µl of 5 X enzyme buffer (Cinnagen, Tehran, Iran) and 1 µl (200 U) of Moloney Murine Leukemia Virus (M-MulV) reverse transcriptase (Revert Aid, Fermentas, Litvany) in a total volume of 20 µl. The reaction was incubated at 42°C for 1 h followed by a brief centrifugation and then inactivation of the enzyme by heating at100°C for 10 min.

RT-PCR amplification: To amplify the gene encoding the microneme protein 5 (MIC5) of *E. necatrix*, primers Mic5-F (5'-GCACTGCAGGAAATGGGAAG) and the Mic5-R (5'-TTCCCAGAAGCCAAGGTGAA) were designed based on a single EST (accession number EU335049) of E. *necatrix* encoding microneme 5 and synthesized by Cinnagen Co. Ltd. Searching of NCBI database revealed that this EST is the only sequence of *Eimeria necatrix* which encodes the protein. The PCR conditions were as follows: initial denaturation at 95°C (2 min), followed by 35 cycles of denaturation at 94°C (40 sec), annealing at 60°C (1 min) and extension at 72°C (90 sec), with a final extension at 72°C (10 min). Amplicon, expected to be 758 bp, was electrophoresed in a 1% agarose gel and visualized by staining with ethidium bromide.

DNA sequence analysis: PCR product was excised from the gel, purified by an extraction kit (QIAgen, Iran) and sequenced by primers Mic5-F and Mic5-R from both ends using a dideoxy termination method in an automated DNA sequencer. Sequence similarity analysis against GenBank database entries was performed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov). We used online tool software at the Expasy website (http://expasy.org/tools) for nucleotide sequences to translate into the corresponding amino acids, and the predicted signal peptide sequence was identified using online tool software at the Expasy website http://www.cbs.dtu.dk/services/SignalP/. A motif search was conducted using the Motif Search Software (http://pfam.sanger.ac.uk/search/sequence). Multiple sequence alignments were obtained by using the CLUSTAL_W program and edited with the BOXSHADE software (http://www.ch.embnet.org/software/BOX_form.html). The CDD-Search software from the NCBI site was used to determine the conserved domains (Marchler-Bauer *et al.*, 2003). Primer sets were generated using Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The Sequence was further analysed using phylogram which is presented in the website http://www.phylogeny.fr.

Results

Sporulated oocysts of *Eimeria necatrix* , before and after breakage are shown in Fig 1 and Fig 2 respectively. Starting with 5×10^5 of oocysts, 365 µg of total RNA was obtained. To amplify the mRNAs encoding MIC5, cDNA was synthesized by using a modified oligo (dT). Thereafter, PCR was performed with a specific primer pair (Mic5-F and Mic5-R). PCR was expected to result in the amplification of a DNA band of 758 bp. Electrophoresis of the PCR product revealed that the expected band was successfully amplified (Fig 3). The purified PCR products of separate reactions were sequenced by the dideoxy chain termination method. The amplified cDNA represents a partial sequence of microneme 5 from the Iranian *Eimeria necatrix* (EnMIC-5).

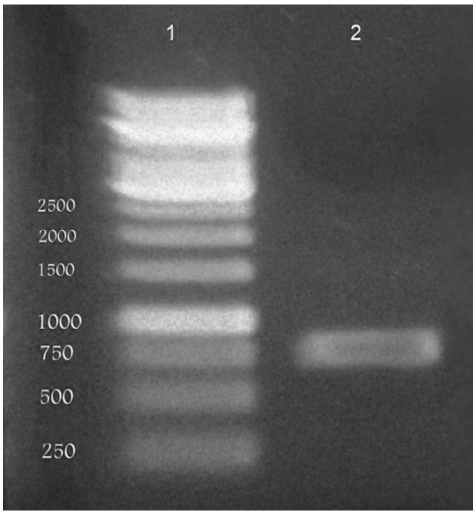


Figure 3: Agarose gel electrophoresis of RT-PCR products from EnMic5 gene isolated from the Iranian *Eimeria necatrix*. Lane 1: DNA size marker. Lane 2 is RT-PCR amplification products. Each lane was loaded with 8 µl of the total reaction.

The amino acid sequence of EnMIC-5 was aligned with those protein sequences reported for *E. tenella* (CAB52368) and the only EST sequence available in the Database *Eimeria necatrix* LZ strain (EU335049) as shown in Fig 4. Multiple alignment of EnMIC-5with those microneme proteins using the CLUSTAL_W program revealed a high degree of primary structural similarity. A high degree of similarities (92% and 91%) were revealed by comparing the amino acids sequence of EnMIC-5 with those reported for *Eimeria tenella* (Et) and *Eimeria necatrix* LZ strain (EU335049), respectively. However, the sequence identity at the nucleotide level was 97% for both sequences. The sequence displayed between 27-35% identity with microneme protein from *Toxoplasma gondii* (EEE21754), micronemal protein MIC4 (Toxoplasma gondii) (ACY68631), goosefish kalliklectin (*Lophiomus setigerus*) (BAG66037), unnamed protein product (*Tetraodon nigroviridis*) (CAF99456), calcium binding EGF domain-containing protein from Cryptosporidium muris RN66 (XP_002140997) and Coagulation factor XI precursor (*Osmerus mordax*) (ACO10049) (Fig. 5). Moreover, the homology is higher at nucleotide level than that at amino acid level. The large blocks of conserved amino acid sequences are scattered throughout the open reading frame (ORF), although the vast majority of identity is located within the One-third of the N-terminus part of the sequences (Fig. 4).

The regions encoding the mature toxin depicted in Fig. 4 in addition to 5 sequences including microneme protein from *Toxoplasma gondii* (EEE21754), micronemal protein MIC4 (*Toxoplasma gondii*) (ACY68631), goosefish kalliklectin (*Lophiomus setigerus*) (BAG66037), unnamed protein product (*Tetraodon nigroviridis*) (CAF99456) and Coagulation factor XI precursor (*Osmerus mordax*) (ACO10049) were aligned and then used to construct a phylogenetic tree (Fig. 5). Those sequences fell into two well-defined groups which are phylogenetically distant despite their primary sequence similarity. The tree shows that the isolated sequence EnMic5 relating to the Iranian *E. necatrix* (En), *E. tenella* (Et1) and the only EST sequence from *E. necatrix* LZ strain (En-EST) were placed, as expected, in the same group. The tree also showes more similarity between *E. necatrix* LZ strain (En-

EST) and *E. tenella* (Et1) rather than that our isolated (Enmic5) (Fig. 5). The rest of the sequences were more closely related to each other as the second group.

Domain analysis of *E. necatrix* protein using SBASE online software showed e-value of 3.76e-08 with conserved domain of PAN/APPLE superfamily and APPLE_Factor_XI_like domain (cd01100) between amino acid resides 130 to 201. This domain has strong similarity to the adhesive plasma pre-kallikrein.

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En        1 -----------------------ALQEMGSEVETAEECQLLCQATSGCEVYSFLGSACKL
En(EST)   1 -----------------------ALQEMGSEVETAEECQLLCQATSGCEVYSFLGSACKL
Et1     481 YNFISGPRQCAGCSTNGSEYSGKALQEMGSEVETAEECQLLCQATSGCEVYSFLGSACKL

En       38 LGSGETKKNVLATSGVKYCTGVCDILGFRAPRREFGYALLLKNKSLDQCRKACAADKKCT
En(EST)  38 LGSGESKKNVLATSGVKYCTGVCDILGFRAPRREFGYALLLKGKSLEDCRKACSADQKCT
Et1     541 LGSGESKKNVLATSGVKYCTGVCDILGFRAPRREFGYALLLKGKSLEDCRKACSADQKCT

En       98 NFTSWLNGDCYVKDDQDHRMQEPLPEATTGWLSCSTCFRQGVGYKATEANLLWTLPTENA
En(EST)  98 NFTSWLNKECYIKDDQDHRMQEPIPEATSGWVSCSTCFRQGVGYKATEANLLWTLPSENA
Et1     601 NFTSWLNKECYIKDDQDHRMQEPIPEATSGWVSCSTCFRQGVGYKATEANLLWTLPSENA

                          PAN/APPLE-like domains

En      158 EECGQRCEAMESCGRFSYDAASKACSMLSGEGEDVEGENLVSGPPWCTRRDTCYQDGVSF
En(EST) 158 EECRQRCEVMESCGRFSYDAASKACSMLSGEGEEVQGENLVSGPPRCARRDTRYQNGVSF
Et1     661 EECRQRCEVMESCGRFSYDAASKACSMLSGEGEEVQGENLVSGPPRCTRRDTCYQNGVSF

En      218 TGGKAISEAEAASSQACQALCEKDAKCRFFTLASG-------------------------
En(EST) 218 TGGKAISEAKAASSQACQELCEKDAKCRFFTLASG-------------------------
Et1     721 TGGKAISEAKAASSQACQELCEKDAKCRFFTLASGKCSLFADDAALRPTKSDGAVSGNKR
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Fig 4: Multiple sequence alignment of EnMIC-5 from the Iranian *E. necatrix* and other microneme proteins. The amino acid sequence of EnMIC-5 (En) was aligned with those reported for *E. tenella* (CAB52368) and the only EST sequence available in the Database *E. necatrix* LZ strain (EU335049). The amino acids are denoted by one-letter symbols. The position of PAN/APPLE-like domain is underlined. Shading indicates identity (black) or conservative substitutions (grey) relative to EnMIC-5. Gaps represented by dashes were introduced to maximize the alignment.

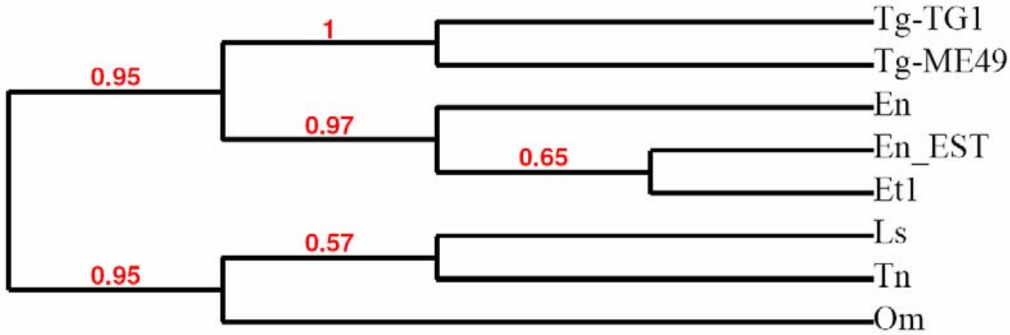


Fig 5: Phylogenetic tree of the EnMic5 (En) from the Iranian *E. necatrix* and other microneme protein counterpart sequences. Phylogeny was reconstructed based on the regions encoding the protein depicted in Fig. 3 in addition to other microneme counterpart sequences including PAN domain-containing protein, putative from Toxoplasma gondii GT1 (Tg-TG1) (EEE21754), micronemal protein MIC4 (Tg-ME49) (*Toxoplasma gondii*) (ACY68631), microneme protein 5 from *E. tenella* (Et1) (CAB52368), goosefish kalliklectin (Ls) (*Lophiomus setigerus*) (BAG66037), unnamed protein product (Tn) (*Tetraodon nigroviridis*) (CAF99456) and Coagulation factor XI precursor (Om) (*Osmerus mordax*) (ACO10049). Numbers in nodes indicate the correlation coefficients between groups.

Discussion

Invasion of host cells is an essential step in the establishment of extra cellular motile zoites of *Eimeria* (sporozoites and merozoites) and is accompanied by the secretion of proteins from specialised apical organelles. Micronemes are the smallest of the apical organelles, structurally and functionally conserved in all apicomplexans (Bumstead and Tomley, 2000). Proteins they secrete promote the attachment of parasites to the potential host cells (Tomley *et al.,* 1996 and Tomley and Soldati, 2001) and motility (Sultan *et al.,* 1997) and thus play a crucial role in the invasion process of apicomplexan parasites.

Micronemes contain around 10 abundant proteins (Kawazoe *et al.*, 1992), and a number of the genes that encode *Eimeria necatrix* micronemal proteins including EnMIC-2 (Qin ZongHua *et al.*, 2005) and EnMIC-5 (Cai Xue peng *et al.,* 2008) have been cloned and characterized.

For molecular biologists working with eimerian parasites, isolation of appreciable amounts of high-quality RNA, the cornerstone of RNA-based molecular techniques, has often proved challenging. Understanding the limitations of various RNA isolation methods, we isolated total RNA from sporulated oocysts of *E. necatrix* using a method that is rapid, inexpensive and produces a good yield named acid-phenol extraction procedure. This method was compared with ultracentrifugation of GTC lysate with CsTFA and with TRIzol reagent by Johnston D.A et.al (1998). They reported Owing to its simplicity and omission of ultracentrifugation, the acid-phenol method is rapid and cost-effective and permits the simultaneous preparation of numerous samples while abrogating RNA loss and degradation. They extracted the total RNA from 1 gram (ca 1.62×10^8) oocysts yielded 492 µg 0f RNA while we showed this method is effective enough to yield an appropriate RNA (365 µg) from significant lower number of oocysts (5×10^5). Our strategy for isolating EnMIC-5 from sporozoites in Khuzestan province of Iran was successful and sequence comparison at both level of nucleotides and amino acids revealed that the amplified cDNA is highly homologous with MIC-5 of *Eimeria necatrix* LZ strain identified by Cai Xue peng *et al.* (2008) and *Eimeria tenella* but similarity between these two last *Eimeria* is more than our isolate. Although the reasons for this are not clear, they may be due to strain differences or mutations, one could speculate that microneme sequence of *E. necatrix* of this region (Khuzestan) may represent a new family of microneme proteins with apple domain like motifs.

Apple domains, which are a subset of the plasminogen, apple, nematode (PAN) superfamily, have been identified in plasminogen-related proteins such as coagulation factor XI, plasma pre-kallikrein, hepatocyte growth factor, macrophage stimulation factor and also in several nematode proteins. Apple/PAN domains have three conserved disulphide bridges that are essential for their tertiary structure, but homology in the primary amino acid sequence between domains is generally low, which may contribute to their very different and highly specific ligand binding properties (Carruthers and Tomley, 2008). As it was showed in Fig. 4, the presence and spacing of cysteine residues are rigorously conserved in all mentioned molecules, indicating their importance for the structure and/or function of the microneme proteins via disulfide bond formation

In conclusion, our isolated EnMIC-5 is structurally similar to EnMIC-5 LZ strain identified by Cai Xue peng *et al.* (2008) but there is some allelic polymorphism represents the ability of apicomplexans to generate genetic diversity, using a variety of molecular mechanisms including allelic polymorphism, antigenic variation and genetic recombination.

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تعیین توالی بخشی از cDNA کد کننده ی پروتئین میکرونم ۵ *آیمریا نکاتریکس* جداشده از استان خوزستان

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چکیده

میکرونم ها اندامک های ترشحی موجود در مراحل تهاجمی انگل های اپی کمپلکسا بوده و حاوی پروتئین هایی هستند که نقش مهمی را در حرکت انگل و تهاجم به سلول های میزبان به عهده دارند.حتی با وجود اینکه هر هفت گونه مهم *آیمریا* و یا ترکیبی از آنها می تواند منجر به بروز کوکسیدیوز گردد، اغلب تحقیقات مولکولی در مورد شناسایی ژنهای میزبان و انگل و پروتئین ها، منحصر به *آیمریا تنلا* می باشد. مطالعه حاضر، جدا سازی و خالص سازی اُاُسیست های *آیمریا نکاتریکس* را با یک روش کم هزینه و آسان برای استخراج RNA کل جهت تعیین توالی بخشی از cDNA کد کننده پروتئین میکرونم ۵ توصیف می کند. برای ساخت این cDNA ، از RNA کل استخراج شده به عنوان الگو، و از الیگو دی تی به عنوان آغازگر استفاده شد. برای تکثیر این cDNA، روش RT-PCR و با استفاده از آغازگرهای اختصاصی برگرفته از توالی (EST) موجود در بانک ژنی انجام شد که به دنبال آن، قطعه ای با وزن ۷۵۸ bp به صورت تک باند و واضح در الکتروفورز مشاهده شد. با آنالیز دامین این قطعه ژنی (EnMIC-5) مشخص شد که این توالی دارای دامین محفوظ متعلق بـه ابـر خانواده Apple/PAN در بین اسید آمینه های ۱۳۰ تا ۲۰۱ بوده که بسیار شبیه به پری کالی کرئین پلاسمایی چسبنده می باشد. علی رغم تشابه توالی قطعه مزبور با توالی موجود در بانک ژنی، تفاوت های موجود می تواند بیانگر تعدادی پلی مورفیسم آ للی باشد.

واژگان کلیدی: *آیمریا نکاتریکس*، میکرونم، RT-PCR